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Genotyping, identification and multifunctional features of yeasts associated to Bosana naturally black table olive fermentations

Cinzia Porru [a], Francisco Rodríguez-Gómez [b], Antonio Benítez-Cabello [b], Rufino Jiménez-Díaz [b], Giacomo Zara [a], Marilena Budroni [a, *], Ilaria Mannazzu [a], Francisco Noé Arroyo-López [b]

[a] Dipartimento di Agraria, University of Sassari, Viale Italia 39, Sassari, I-07100, Italy
[b] Food Biotechnology Department, Instituto de la Grasa (CSIC), University Campus Pablo de Olavide, Building 46. Crta. Utrera Km 1, 41013, Seville, Spain

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ABSTRACT

Directly brined black table olives of Bosana variety are a traditional food product of Sardinia island (Italy), spontaneously fermented by yeasts among other microorganisms. However, as far as we know, the identification, biotechnological and probiotic potential of this yeast community has not been investigated yet. In this work, a total of 72 yeast isolates previously obtained from Bosana olive brines were first genotyped by Random Amplified Polymorphic DNA (RAPD-PCR) analysis with primer M13, and then identified by sequencing of D1/D2 domains of rDNA 26S gene. The dominant species were *Wickerhamomyces anomalus* and *Nakazawaea molendini-olei*, albeit *Candida diddensiae, Candida boidinii, Zygotorulaspora mrakii,* and *Saccharomyces cerevisiae* were also present in lower proportions. For the different biotypes of yeasts obtained, the multivariate analysis of their technological (esterase, lipase and β-glucosidase activities, growth in presence of oleuropein, resistance and susceptibility to NaCl) and probiotic (removal of cholesterol, gastric and pancreatic digestions, biofilms assays alone and in co-culture with *Lactobacillus pentosus*) features, showed that *W. anomalus* Wa1 exhibited the best technological characteristics, while *S. cerevisiae* Sc24 and *C. boidinii* Cb60 showed promising probiotic features. Therefore, they may have potential application as multifunctional starters, alone or in combination with lactic acid bacteria, during olive processing, albeit further studies are necessary to validate these results.

1. Introduction

Table olives are one of the most important and well-known fermented vegetables of the food industry, with an estimated world production above 2.5 million tonnes per year (IOC, 2017). The main producing area is around the Mediterranean Basin, with an outstanding contribution of the European Union (EU) (around 30% of production). Within it, Spain is the leading country, followed by Greece and Italy (IOC, 2017). The Mediterranean diet pyramid recommends a daily intake of table olives due to the nutritional benefits associated with this fermented vegetable (Bach-Faig et al., 2011; Arroyo-López et al., 2016). Particularly, consumers are increasingly demanding more traditional and natural homemade olive preparations.

Olive fruit contains a bitter component (oleuropein) that makes it unpalatable. Thus, the main goal of table olive processing is to remove the natural bitterness of fresh fruit. In the case of natural olives, not subjected to "lye" treatment as occur in Bosana olive specialty, debittering is obtained just by immersion of the harvested drupes in brine. In this case, the removal of the fruit bitterness is produced by hydrolysis of oleuropein, due, among other factors, to esterase or β-glucosidase activities by microorganisms (Tassou et al., 2002; Arroyo-Lopez et al., 2012), or by diffusion of this compound from fruits to the surrounding brine (Garrido-Fernandez et al., 1997). Therefore, the process is rather slow.

Lactic acid bacteria (LAB) play an important role in table olive fermentations (Garrido-Fernandez et al., 1997). In fact, they produce antimicrobial substances (bacteriocins), and lactic acid originated from the fermentation of sugars, resulting in pH decrease that enhances table olives safety (Ruiz-Barba and Jimenez-Diaz, 1995; Corsetti et al., 2012). Especially, *Lactobacillus pentosus* is a

* Corresponding author. Dipartimento AGRARIA, University of Sassari, Italy.
E-mail address: mbudroni@uniss.it (M. Budroni).

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species with a significant relevance in table olive production because of its predominance during fermentative process and use as starter culture (Tofalo et al., 2014; Martorana et al., 2017; Rodríguez-Gómez et al., 2017). However, sometimes, yeasts can be the dominant microorganism during table olive fermentation, especially when LAB are inhibited due to the presence of phenolic compounds or the high salt and low pH levels obtained during the fermentation process (Ruiz-Barba et al., 1993; Garrido-Fernandez et al., 1997). Thereby, they have also a considerable influence during olive processing, acting as desirable or undesirable microorganisms (Arroyo-Lopez et al., 2012). For many years, the search for starters with application in olive fermentation was focused exclusively on the activity of LAB. However, in the last years, different works and reviews have considered the beneficial effects that the use of yeasts may have during olive processing due to their biotechnological and probiotic potential (Silva et al., 2011; Arroyo-Lopez et al., 2012; Bevilacqua et al., 2012, 2013, 2015; Tofalo et al., 2013; Bleve et al., 2015; Bonatsou et al., 2015; De Angelis et al., 2015; Tufariello et al., 2015). For this reason, yeasts may be exploited in table olive processing as multifunctional starters, particularly in the case of natural olives which spontaneous processing is usually performed by a microbiota mainly composed of yeasts (Garrido-Fernandez et al., 1997).

The present paper has the double objective of i) expanding the knowledge of the yeast community associated to traditional table olives preparations from Sardinia island, and ii) determining their biotechnological and probiotic potential for their use as multifunctional starters.

2. Material and methods

2.1. Strains isolation

The study was conducted with a collection of 72 yeasts previously isolated from brines of traditional spontaneous fermentations of Bosana fruits (Pinna, 2005), an olive variety largely cultivated in Northern Sardinia (Italy). Fruits were harvested during the 2003/2004 season at black ripening stage in the same olive ground. After sorting, sizing, and washing, the olives were dried and placed into barrels (20 L capacity), which were filled with 10.5 kg of fruits and 9.5 L of 60 g/L NaCl brine (ratio brine/drupe of approximately 1:1). Yeasts were isolated as described by Hernández et al. (2007) during the fermentation process at 0, 1, 2, 5, 10, 15, 22, 30, 60, 90, 120, 150, and 180 days. Then, they were stored (−80 °C) in Dipartimento di Agraria of Sassary University (Italy) in YM broth medium (Difco™, Becton and Dickinson Company, Sparks, MD, USA) supplemented with 20% glycerol for further analysis.

2.2. Genotyping and identification of yeast population

Genomic DNA extraction was performed by the procedure described by Senses-Ergul et al. (2012). The extracted and purified DNA was quantified using the internal protocol incorporated in the Spectrostar Nano spectrophotometer (BMG LABTECH, Germany) through absorbance reading at 260 nm.

Yeast genotyping was performed by Random Amplified Polymorphic DNA (RAPD)-PCR analysis with M13 primer according to the procedures described by Andrighetto et al. (2000). RAPD products were visualized with a gel analyzer model EnduroTM GDS (Labnet International, Inc., USA). The fingerprints obtained for the different yeast isolates were captured and analysed using Bionumerics 6.6 software (Applied Maths, Belgium). Dendrogram for clustering comparison was built with UPGMA (Unweighted Pair Group Method) method and Pearson correlation. Reproducibility and sensibility of the technique were evaluated by comparing the profiles of the strain *Wickerhamomyces anomalus* TOMC-Y45 obtained from seven different DNA extractions, PCR and agarose gels.

To corroborate the clustering analysis and for identification of the yeasts at the species level, sequencing of D1/D2 domains of 26S rDNA was performed for all the yeast isolates using NL1 and NL4 primers (Kurtzman and Robnett, 1998) and the protocols described by Arroyo-Lopez et al. (2006). Purification of the PCR products for sequencing was carried out using the Isolate DNA kit (Bioline, Taunton, USA) according to the manufacturer's instructions, and sequenced by the StabVida Sequencing Services (Lisbon, Portugal). The alignment of sequences obtained was performed with software MEGA 5.0 (Molecular Evolution Genetic Analysis) and then compared with sequences deposited in the NCBI (National Center for Biotechnology Information, USA) GenBank database using the BLAST (Basic Local Alignment Search Tool) software.

2.3. Evaluation of technological features

The following technological assays were performed (in triplicate) using one representative strain chosen randomly from each different cluster of the dendrogram: i) the modelling of susceptibility (NIC) and resistance (MIC) to NaCl in YM broth medium (pH 4.5), supplemented with different NaCl concentrations (0, 10, 30, 50, 70, 80, 90, 100, 110, 120, 130, 150, and 180 g/L), was determined by the application of a dose/response model using a modified Gompertz equation according to procedures described by Bonatsou et al. (2015); ii) extracellular and cellular β-glucosidase, esterase and lipase enzymatic activities were measured spectrophotometrically (absorbance at 410 nm) based on the ability of these enzymes to release *p*-nitrophenol from different chromogenic substrate and expressed as nmol ml/h according to procedures described by Rodríguez-Gómez et al. (2012); and iii) growth in the presence of oleuropein was assayed in YNB medium (Difco) at pH 4.5 supplemented with 1.0% (wt/vol) of oleuropein (Extrasynthese, Lyon, France) as the only fermentable substrate. Previously, yeasts were subjected to starvation for 2 h in sterile saline solution (0.9% NaCl) to remove any accumulated nutrients. Growth was monitored at 28 °C for 7 days in an automated spectrophotometer model Bioscreen C (Labsystem, Finland) by OD measurements. Maximum specific growth rate (μ_{max}) in the presence of oleuropein was obtained from yeast growth curves by primarily modelling using the reparametized Gompertz equation proposed by Zwietering et al. (1990). For all tests described above, *Saccharomyces boulardii* was used as internal control.

2.4. Evaluation of probiotic potential

The following probiotic *in vitro* assays were also carried out (in triplicate) to the representative isolates obtained from each different cluster of the dendrogram, using the well-known probiotic yeast *S. boulardii* (Ultra Levura, Zambon S.A.U, Barcelona, Spain) as control: i) sequential simulated gastric and pancreatic digestion was performed according to the procedure described by Bonatsou et al. (2015), ii) the ability to remove cholesterol was determined in YNB as basal medium supplemented with cholesterol (182 mg/L) + glucose (10 g/L), cholesterol + glucose + oxgall (3 g/L) (Sigma-Aldrich), and cholesterol + oxgall, previously subjecting yeasts to starvation in 0.9% sterile saline solution for 2 h. The purpose of employing three different media was to determine if the presence of different compounds could stimulate or inhibit cholesterol removal by yeasts. Cholesterol was quantified by absorbance at 500 nm and further comparison with a previous calibration curve (data not shown). All reagents were included in the Biosystem Kit (Barcelona, Spain); iii) biofilms assays on abiotic surfaces was carried out with the protocol described by Toledo-